Hydrobiologia **357:** 139–153, 1997.

International study on *Artemia*. LIII. Morphological study of *Artemia* with emphasis to Old World strains. I. Bisexual populations

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Received 20 July 1995; in revised form 20 August 1995; accepted 20 October 1995

Key words: Artemia, morphology, morphometry, discriminant analysis, cluster analysis, scanning electron microscopy

Abstract

A detailed morphological and allometrical study was performed with adult males and females of eleven bisexual populations of brine shrimp *Artemia*. Multivariate procedures, discriminant and cluster analysis, allowed to separate and group together populations which exhibit great genetic similarities. The eleven populations studied form four distinct groups: the *A. franciscana* group, the *A. tunisiana* group, the *A. urmiana* group and a broader group which includes Eastern Old World populations. Scanning electron microscopy revealed differences in the male genital organs of an *A. tunisiana* population by lacking a medial protuberance in the base of the penes while the pattern of the ectodermal ridges of the brood pouch of *A. urmiana* markedly differed from the other populations studied.

Introduction

The brine shrimp *Artemia* is a cosmopolitan Anostracan crustacean. The genus is a complex of species and superspecies defined by the criterion of reproductive isolation (Browne & Bowen, 1991). The means to approach the various populations taxonomically and to classify them into biological species are the study of cytogenetics, anatomical characters and electrophoretic data (Beardmore & Abreu-Grobois, 1983).

Many scientists are considering the genus *Artemia* as a complex of sibling species although many studies show that there are morphological differences among the individuals of different species (Amat, 1980; Hontoria & Amat, 1992a,b; Pilla, 1992; Triantaphyllidis et al., 1995). Comparative morphological characterization has mostly been performed on females. Recently, Pilla (1992) and Pilla & Beardmore (1994) showed that male morphometric characters can be at least as informative.

It will soon be possible to extend our present knowlegde on the biogeography and evolution of the genus as several new strains from Asia are becoming available for study, especially from the People's Republic of China (P.R. China), Kazakhstan and Iran. In this paper we perform a morphological study of *Artemia* populations that belong to well-described species and compare them with populations that are taxonomically still not well characterized. The use of multivariate procedures has been employed to test the efficiency of these methods and create a data matrix helpful to classify future samples of unknown origin or those taxonomically problematic.

140

Table 1. List of the populations studied, their *Artemia* Reference Center (ARC) cyst bank code number and abbreviations used

Population	ARC cyst bank code number	Abbreviation
San Francisco Bay, California, USA	1209	SFB
Great Salt Lake, USA, 'Argent I grade'	1286	GSLI
Great Salt Lake, USA, 'Argent Pt grade'	1287	GSLPT
Yuncheng, Shanxi, P.R. China	1218	YUN
Yimeng, Inner Mongolia, P.R. China	1188	YIM
Inner Mongolia, unknown locality, P.R. China	1154	INM
Haolebaoji, Inner Mongolia, P.R. China	1215	HAL
Kazakhstan, unknown locality	1039	KAZ
Sfax, Tunisia	1269	SFA
Mégrine, Tunisia	1268	MEG
Urmia Lake, Iran	1230	URM

Materials and methods

In this study eleven bisexual populations of *Artemia* were examined. Table 1 displays the origin of each population and the abbreviations that are used.

Culture conditions

Cysts from each sample were incubated in artificial 0.45 μm-filtered 35 ppt Dietrich & Kalle medium (D&K) (Kalle, 1971) which was prepared following the modifications of Vanhaecke et al. (1984). Hatching conditions were according to Sorgeloos et al. (1986). After 30 hours of incubation (when maximum hatching percentage was obtained) nauplii were transferred to one liter cylindroconical glass tubes containing 0.45 μm-filtered D&K medium of 50 ppt salinity. The initial number of naupli per tube was 200. Initial density was one animal per 2 ml while from day 8 onwards it was reduced to one animal per 4 ml. The animals were kept under mild aeration, at 25 ± 1 °C with 12 hr cool white fluorescent lighting daily. Each population had three replicates and the animals were fed on a mixed diet of the alga *Dunaliella tertiolecta* Butcher and the yeast-based formulated feed LANSY PZ from INVE Aquaculture SA, Belgium, following the feeding schedule of Triantaphyllidis et al. (1995). Survival was recorded for adaptation of the feeding schedule at each water renewal i.e. on day 8 and every three to five days thereafter until the animals reached the adult stage and started to reproduce. Survival ranged between 62 ± 11% and 88 ± 8% for the populations studied.

Biometry

The animals were examined morphologically when most of them reached the adult stage (i.e. when offspring production observed). The various populations required different periods for maturation and offspring release. New World populations were evaluated after about 30 days while some Old World populations required a longer culture period to reach the same developmental stage (about 40 days). In this way we evaluated animals that had released one to three broods.

The following morphological parameters were examined on at least 30 individuals per sex per population: total length, abdominal length, length from the third abdominal segment to the end of the abdomen, length of the eighth abdominal segment, width of the head, width of the third abdominal segment, length of furca, number of setae on each branch of the furca, length of the first antenna, maximum distance between the compound eyes and maximum diameter of the compound eye. In addition, the width of the ovisac was measured in females. The animals were anaesthetized in chloroform-saturated seawater (Gilchrist, 1960) and measured under a dissection microscope equipped with a camera lucida and then the data were analyzed with a digitizer.

Statistical analysis

Morphological variables were log transformed while meristic variables such as the number of setae on the left and right branch of the telson were square-root

transformed in order to increase homogeneity of variances (Sokal & Rohlf, 1981; Pilla, 1992).

Data for each population were tested for fitting to the normal distribution by applying the Chi-square and Kolmogorov-Smirnov tests (Sokal & Rohlf, 1981). The homogeneity of variances was tested using the Levene's test (Norusis, 1993). The means were compared using one-way analysis of variance (Anova) when variances were homoscedastic by employing the Tukey-B test (Norusis, 1993), while an approximate test of equality of means of Games & Howell (1976) was conducted when variances were heteroscedastic.

Morphological data were processed with discriminant function analysis (Tatsuoka, 1971; Pimentel, 1979; Sokal & Rohlf, 1981; Hontoria & Amat, 1992a,b); this multivariate procedure computes a series of new variables $(Z_1, Z_2, \ldots)$ which are linear functions of the morphological parameters considered with the form $Z_n = l_1X_1 + l_2X_2 + \ldots$ (where l_n are the calculated discriminant coefficients and X_s the variables being considered). This discriminant function is constructed in such a way that as many as possible members of one population have high values for Z and as many as possible members of the other have low values, so that Z serves as a much better discriminant of the populations than variable X_1 or X_2 does taken singly (for more details see Tatsuoka, 1971; Pimentel, 1979; Sokal & Rohlf, 1981). Discriminant analysis was carried out separately on males and females, while the origin of each population was used as separation criterion. A stepwise variable selection method was applied based on the criterion of minimizing Wilks' lambda. Thus, at each step the variable that minimizes the overall Wilks' lambda is entered. Each entry or removal of a variable is considered a step.

The single linkage method (nearest neighbor technique) was used to construct dendrograms for identification of the morphologically homogeneous groups (clusters). The first two cases combined are those with the smallest distance, or greatest similarity, between them (Norusis, 1993). The measure of selection was based on the squared Euclidean distance between the populations and the data were standardized to Z scores, with a mean of 0 and a standard deviation of 1 (for more details on cluster analysis see Pimentel, 1979; Romesburg, 1984; Norusis, 1993).

All the above calculations were performed with the statistical packet SPSS (release 6.0 for Windows), run on the mainframe computer of the University of Ghent.

Scanning electron microscopy

For the scanning electron microscopy study adults were fixed overnight in a glutaraldehyde-paraformaldehyde mixture (Karnovsky, 1965) diluted 3 : 1 with cacodylate buffer 0.2 M, pH 7.4, rinsed in cacodylate buffer and postfixed in 2% osmium tetroxide in cacodylate buffer. Dehydration in acetone was followed by critical point drying. After mounting the specimens they were gold coated and examined with an ISI-SR-50 scanning electron microscope (SEM).

Results

Males

The male morphological characters follow the normal distribution as Chi-square and Kolmogorov-Smirnov tests gave no statistically significant results ($P > 0.05$). Levene's test revealed homoscedastic variances for the abdominal length and the length of the eighth abdominal segment, while all the other parameters showed departures from homoscedasticity. Table 2 shows the results of the measurements for the males. The F ratios (which are the between groups mean square to the within groups mean square) and their significance revealed highly statistical significant differences among the means of the morphological parameters of the various populations ($P < 0.00005$). The characters that present the higher variability are the length from the end of the eighth abdominal segment to the third abdominal segment and the abdominal length as well as the length of the furca and the number of setae in the furca. Table 3 summarizes the results of the Anova and of the statistically significant interpopulation differences (method of Games & Howell, 1976) for the morphological variables studied.

Discriminant analysis based on the origin of each population as a separation criterion resulted in 10 canonical discriminant functions after 24 steps. The first seven functions are highly statistical significant ($P < 0.0001$) but the last three are not ($P > 0.05$). The first five discriminant functions give a cumulative separation percentage of 98.17%, while the first three that appear in Table 4 contribute to a separation of 94.26%. Table 4 shows the discriminant coefficients (standardized and unstandardized) for the males. More detailed information on the results of the classification phase for the males is given in Table 5A.

142

Table 2. Mean values (standard deviation in parenthesis) of morphometric and meristic characters of males of different bisexual *Artemia* populations. n = number of animals analysed. Abbreviations of populations in Table 1. A: total length, B: abdominal length, C: length from the third abdominal segment to the end of the abdomen, D: length of the eighth abdominal segment, E: width of third abdominal segment, F: length of furca, G: width of head, H: length of first antenna, I: distance between eyes, J: diameter of complex eye, K: number of setae on the left branch of the furca, L: number of setae on the right branch of the furca. Wilks' lambda (λ) is given by the equation $\lambda = 1 - \eta^2$, where η^2 is the ratio of the between-groups sum of squares to the total sum of squares and represents the proportion of the total variance attributable to differences among the groups (Norusis, 1993)

	SFB $n=30$	GSLI $n=32$	GSLPT $n=31$	YUN $n=30$	YIM $n=31$	INM $n=29$	HAL $n=30$	KAZ $n=30$	SFA $n=30$	MEG $n=31$	URM $n=30$	F ratio	Wilks' lambda	F Probability
A	8.75	9.22	8.41	10.44	10.77	10.83	10.28	10.16	8.86	9.32	12.37	92.2330	0.25937	< 0.00005
	(0.50)	(0.41)	(0.65)	(0.60)	(0.91)	(0.95)	(0.84)	(0.48)	(0.59)	(0.50)	(0.62)			
B	3.91	4.21	3.76	5.55	5.66	5.71	5.30	5.21	4.66	5.11	7.14	146.3288	0.18082	< 0.00005
	(0.40)	(0.31)	(0.38)	(0.43)	(0.56)	(0.64)	(0.47)	(0.34)	(0.36)	(0.34)	(0.46)			
C	3.14	3.40	3.01	4.54	4.74	4.77	4.28	4.31	4.00	4.37	6.22	156.4394	0.17114	< 0.00005
	(0.34)	(0.27)	(0.35)	(0.37)	(0.52)	(0.56)	(0.40)	(0.31)	(0.32)	(0.30)	(0.44)			
D	0.97	0.97	0.94	1.34	1.32	1.30	1.28	1.29	1.29	1.48	1.86	101.7097	0.24103	< 0.00005
	(0.14)	(0.10)	(0.12)	(0.15)	(0.14)	(0.17)	(0.14)	(0.12)	(0.14)	(0.12)	(0.14)			
E	0.71	0.63	0.67	0.59	0.58	0.56	0.61	0.62	0.47	0.50	0.51	80.8501	0.28546	< 0.00005
	(0.04)	(0.05)	(0.05)	(0.03)	(0.06)	(0.06)	(0.04)	(0.04)	(0.04)	(0.03)	(0.03)			
F	0.31	0.27	0.31	0.45	0.39	0.36	0.52	0.52	0.39	0.43	0.18	134.4511	0.19370	< 0.00005
	(0.05)	(0.05)	(0.04)	(0.06)	(0.05)	(0.06)	(0.07)	(0.04)	(0.06)	(0.05)	(0.03)			
G	0.98	0.91	0.98	0.93	0.96	0.93	1.00	0.98	0.86	0.87	0.97	17.2853	0.65140	< 0.00005
	(0.05)	(0.04)	(0.06)	(0.05)	(0.14)	(0.08)	(0.06)	(0.06)	(0.05)	(0.03)	(0.05)			
H	1.30	1.38	1.29	1.49	1.65	1.61	1.70	1.71	1.25	1.33	1.56	61.6430	0.34383	< 0.00005
	(0.09)	(0.10)	(0.13)	(0.09)	(0.18)	(0.11)	(0.13)	(0.13)	(0.11)	(0.08)	(0.14)			
I	1.93	1.96	1.93	1.96	2.03	1.99	2.00	2.07	1.73	1.81	2.14	29.2691	0.52461	< 0.00005
	(0.11)	(0.08)	(0.12)	(0.09)	(0.16)	(0.12)	(0.16)	(0.09)	(0.12)	(0.09)	(0.14)			
J	0.39	0.41	0.39	0.39	0.41	0.40	0.42	0.42	0.37	0.40	0.45	7.1857	0.81802	< 0.00005
	(0.03)	(0.03)	(0.04)	(0.03)	(0.04)	(0.04)	(0.05)	(0.03)	(0.03)	(0.03)	(0.05)			
K	11.00	11.56	11.58	12.67	11.90	10.52	14.77	16.07	8.53	9.68	2.67	111.8192	0.22412	< 0.00005
	(1.51)	(2.71)	(1.71)	(2.50)	(2.13)	(2.29)	(1.81)	(2.08)	(1.63)	(2.33)	(1.56)			
L	10.83	11.78	11.48	13.03	11.52	10.14	14.90	16.63	8.33	9.64	2.87	113.0598	0.22221	< 0.00005
	(1.46)	(2.84)	(1.67)	(2.51)	(2.17)	(1.92)	(2.17)	(1.99)	(1.73)	(2.24)	(1.48)			

Figure 1 shows the plot of the discriminant analysis computed for the males. Figure 2 depicts the results of the cluster analysis. The URM population, representing *A. urmiana*, is discriminated from all the other populations, suggesting that it is a species with large morphological differentiation compared to other *Artemia* species. To this contributes the very long abdomen, the length from the third abdominal segment to the end of the abdomen, the length of the eighth abdominal segment, the large distance between the eyes and their diameter ($P < 0.05$) and the furca which is rudimentary lobed with few setae. There is very good separation of the populations SFB, GSLI and GSLPT, which form the *A. franciscana* group, from the group of *A. tunisiana* which is represented by the SFA and MEG populations. YUN is quite close to INM and YIM populations, while KAZ population presents great similarities with HAL population.

Scanning electron microscopy of the claspers and frontal knobs showed differences between the populations, however, they were difficult to quantify. The external aspect of the penis showed an interesting characteristic: in contrast to all populations studied, the penis of SFA (MEG was not available for scanning electron microscopy) lacks a medial spine-like outgrowth at the basal part (Figure 3). The scanning electron micrograph of Figure 4 gives an example of the differences observed in the number of setae of the furca.

Table 3. Significant differences for the mean values of morphometric and meristic characters of males. The differences determined by Anova and the Tukey's B test (Norusis, 1993) when the Levene's test showed homoscedasticity of the variances; when variances were heteroscadastic the approximate test of equality of means of Games & Howell (1976) was employed. Populations that share the same letter (a, b, . . . , g) per row, are not significantly different (a = 0.05). The asterisk denotes the characters that the Levene's test showed departure from homoscadasticity. The codes (A, B, . . . , L) for the various characters are the same as in Table 2. The abbreviations of populations can be found in Table 1

	SFB	GSLI	GSLPT	YUN	YIM	INM	HAL	KAZ	SFA	MEG	URM
A*	a, b	b, c	a	d, e	e	e	d, e	d	a, b, c	c	f
B	a	b	a	e, f	f	f	d, e	d	c	d	g
C*	a, b	b	a	d, e	d, e	d, e	c, d	c, d	c, d	d, e	f
D	a	a	a	b	b	b	b	b	b	c	d
E*	g	e, f	f, g	c, d, e	c, d, e	c, d, e	d, e, f	e, f	a	a, b	a, b
F*	b, c	b	b, c	e, f	d, e	c, d	f, g	g	d, e	d, e, f	a
G*	c, d, e	a, b	c, d, e	a, b, c, d	a, b, c, d, e	a, b, c	e	d, e	a	a	c, d, e
H*	a, b	a, b, c	a, b	c	d, e	d, e	d, e	d, e	a	a, b	d
I*	b, c	c	b, c	c	c, d	c, d	c, d	d	a	a, b	d
J*	a, b, c	b, c	a, b, c	a, b	b, c	a, b, c	b, c	b, c	a	a, b, c	c
K*	c, d, e	d, e	d, e	d, e	d, e	b, c, d	f	f	b	b, c, d	a
L*	c, d	c, d, e	c, d, e	d, e, f	c, d, e	b, c, d	f, g	g	b	b, c	a

Table 4. Results of the discriminant analysis for males. The classification was based on the origin of each population

Variables	Unstandardized canonical discriminant function coefficients*			Standardized canonical discriminant function coefficients*		
	1	2	3	1	2	3
Total length	0.7095387	–17.1764248	23.0907575	0.02034	–0.49229	0.66180
Abdominal length	7.5956034	10.2769151	15.2798679	0.28287	0.55199	0.48626
Length 3rd abdom. seg.	0.7095387	–17.1764248	23.0907575	0.45137	–0.07786	–0.23914
Length 8th abdom. seg.	1.9206692	0.1249897	–11.4538037	0.09297	0.00605	–0.55440
Width of 3rd abdom. segm.	–22.0141777	–0.8556822	6.0507047	0.45137	–0.07786	–0.23914
Length of furca	3.0205863	11.8580135	–6.1197380	0.19700	0.77335	–0.39912
Width of head	–1.1449499	–0.2271714	–0.80295018	–0.03338	–0.00662	–0.23409
Length of 1st antenna	3.3606315	10.2769151	15.2798679	0.12123	0.37072	0.55119
Distance between eyes	–0.7590271	–15.2893719	–1.1566210	–0.01992	–0.40117	–0.03035
Diameter of eyes	1.0119323	–2.8085595	–4.3327717	0.04181	–0.11605	–0.17904
No. of setae left branch	–0.5056685	0.6930746	0.6384079	–0.16688	0.22872	0.21068
No. of setae right branch	–0.2795333	0.5898596	0.6300687	–0.09115	0.19235	0.20546
Constant	–14.389475	10.4569948	–36.861350			

	Eigenvalue**	Percentage of variance	Cumulative percentage	Canonical correlation	Wilks' lambda***	Chi-square	DF	*P*
Function 1	9.0040	45.87	45.87	0.9487	0.001273	2143.245	120	< 0.0001
Function 2	6.6390	33.83	79.70	0.9323	0.012735	1402.837	99	< 0.0001
Function 3	2.8582	14.56	94.26	0.8607	0.097282	749.142	80	< 0.0001

* The unstandardized coefficients are the multipliers of the variables when they are expressed in the original units, while the standardized coefficients are used when the variables are standardized to a mean of 0 and a standard deviation of 1, just like in multiple regression (Norusis, 1993).

** Eigenvalue is the ratio of the between groups to within groups sums of squares. Large eigenvalues are associated with 'good' functions (Norusis, 1993).

*** See Table 2.

Table 5. Classification results of discriminant analysis for males (A) and females (B) showing the percentage of individuals classified in each group. The diagonal elements are the number of cases classified correctly into the groups and serve as an indicator of the effectiveness of the discriminant analysis. The percent of 'grouped' cases correctly classified is 87.72% for the males and 91.94% for the females. The abbreviations of populations can be found in Table 1

(A)

Actual Group	No. of cases	Predicted Group Membership (%)										
		SFB	GSLI	GSLPT	YUN	YIM	INM	HAL	KAZ	SFA	MEG	URM
SFB	30	70.0	0.0	30.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GSLI	32	3.1	96.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GSLPT	31	6.5	0.0	93.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
YUN	30	0.0	0.0	0.0	100.	0.0	0.0	0.0	0.0	0.0	0.0	0.0
YIM	31	0.0	0.0	0.0	9.7	64.5	22.6	3.2	0.0	0.0	0.0	0.0
INM	29	0.0	3.4	0.0	0.0	17.2	79.3	0.0	0.0	0.0	0.0	0.0
HAL	30	0.0	0.0	0.0	6.7	3.3	0.0	83.3	6.7	0.0	0.0	0.0
KAZ	30	0.0	0.0	0.0	0.0	0.0	0.0	3.3	96.7	0.0	0.0	0.0
SFA	30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	90.0	10.0	0.0
MEG	31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.7	90.3	0.0
URM	30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
(B)												
SFB	30	90.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GSLI	32	3.1	96.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GSLPT	31	9.7	0.0	90.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
YUN	30	0.0	0.0	0.0	83.3	16.7	0.0	0.0	0.0	0.0	0.0	0.0
YIM	30	0.0	3.3	0.0	16.7	70.0	3.3	3.3	3.3	0.0	0.0	0.0
INM	30	0.0	0.0	0.0	3.3	6.7	90.0	0.0	0.0	0.0	0.0	0.0
HAL	30	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
KAZ	31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0
SFA	31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	96.8	3.2	0.0
MEG	30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.7	93.3	0.0
URM	30	0.0	0.0	0.0	0.0	0.0	.0	0.0	0.0	0.0	0.0	100.0

Females

Table 6 summarizes the mean values of morphometric and meristic characters of the females. The characters followed the normal distribution (Chi-square and Kolmogorov-Smirnov tests, $P > 0.05$). As in the case of males, F ratios revealed statistically significant differences between the various populations ($P < 0.00005$). The characters that presented the higher variability and thus most significantly contributed to the separation of the various populations were the length from the third abdominal segment to the end of the abdomen and the length of the first antenna; the length of the furca and the meristic characters (namely the number of setae on the left and right branch of the furca) were found to be also characters of high diagnostic value. The diameter of the complex eyes, as in the case of males, was not a character with high variability and so was the width of the ovisac. Levene's test showed that the variances of total length, abdominal length, length from the third abdominal segment to the end of the abdomen, length of the eighth abdominal segment, width of head, diameter of complex eye and width of ovisac were not statistically significant and thus Anova could be employed for these characters ($P > 0.05$). The rest of the characters showed statistically significant differences ($P < 0.05$) in their variances and analysed by the test of Games & Howell (1976). The results are summarized in Table 7.

Discriminant analysis resulted in 10 canonical discriminant functions after 26 steps. The first eight functions are highly statistical significant ($P < 0.0001$) while the last two are not ($P > 0.05$). However, the first five discriminant functions are enough to give a cumulative separation percentage of 97.36%, while the first three that appear in Table 8 give a separation per-

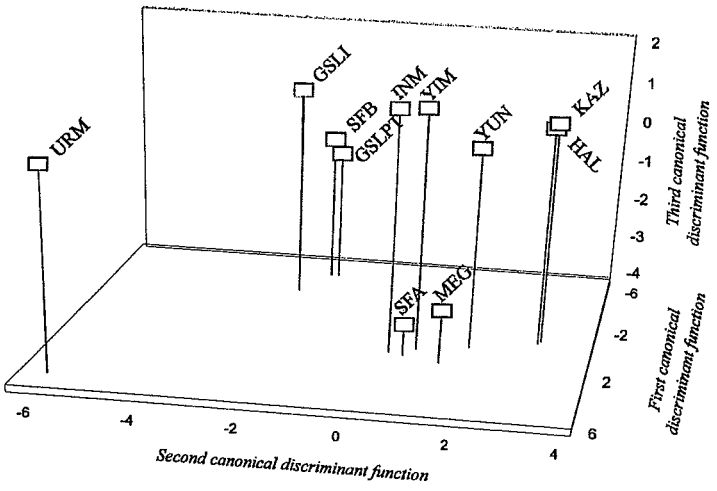

Figure 1. Scatterplot of the first three canonical discriminant functions (group centroids) resulting from the discriminant analysis on males when using the origin of each population as separation factor. For legend to abbreviations see Table 1.

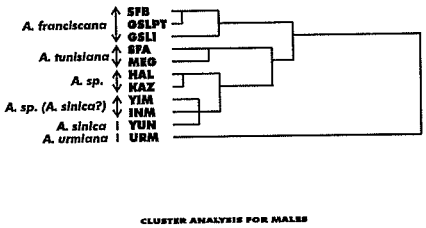

Figure 2. Dendrogram of hierarchical cluster analysis for males using the single linkage method to join clusters (nearest neighbor technique). For legend to abbreviations see Table 1.

centage of 89.97%. Unstandardized and standardized canonical discriminant function coefficients are also presented in Table 8. The classification results are summarized in Table 5B. The overall percentage of correctly classified cases is 91.94%.

Figure 5 displays the plot of the discriminant analysis while Figure 6 depicts the dendrogram of cluster analysis. The URM population is discriminated very far from all the other populations. SFA and MEG, representatives of *A. tunisiana* are very close, as well as SFB, GSLI and GSLPT which form the *A. franciscana* group. The other Eastern Old World populations are forming a broader group. Cluster analysis grouped together YIM, YUN and INM while at a later stage HAL is incorporated into the latter group followed by KAZ in a subsequent stage.

Scanning electron microscopy of the head and uterus showed the same difficulties of quantification as encountered in males. The pattern of the ectodermal ridges of the brood pouch of URM markedly differed from the other populations studied (Figure 7).

146

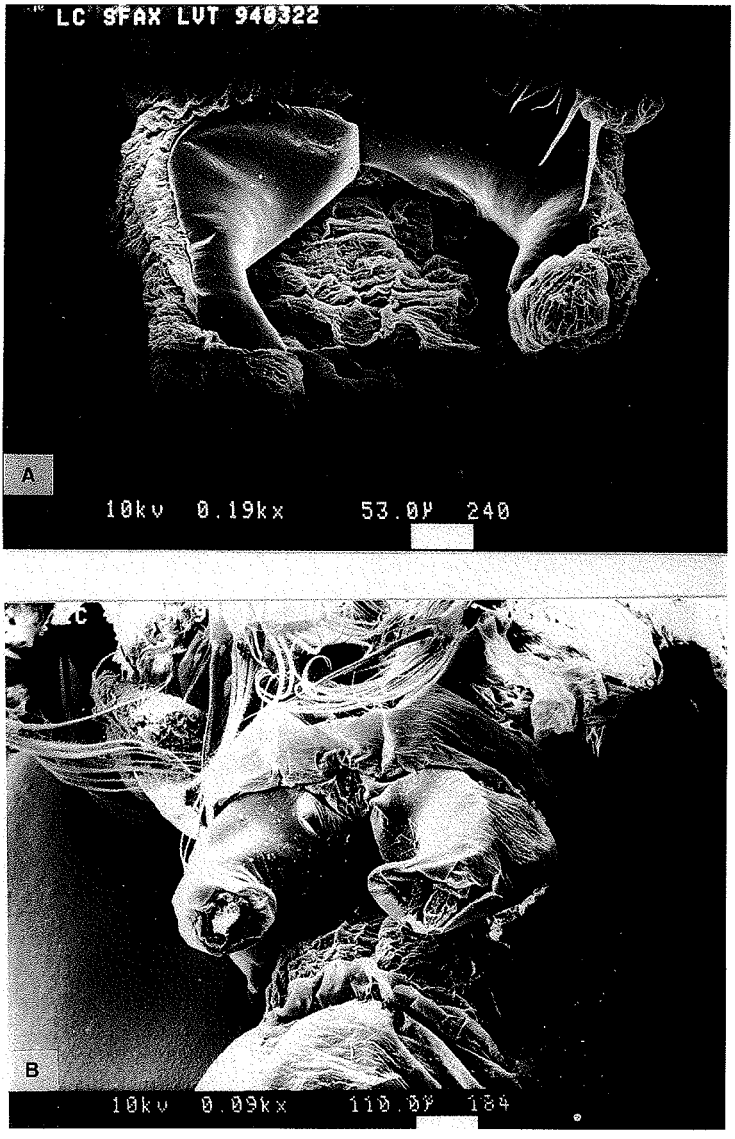

Figure 3. SEM photographs of male genital organs. The external aspect of the penes showed the same variability in most of the cases, but in contrast to all populations studied, SFA (plate A) lacks a spine-like outgrowth at the base of the penis which is very common in all the other populations (plate B shows the protuberances in the SFB population).

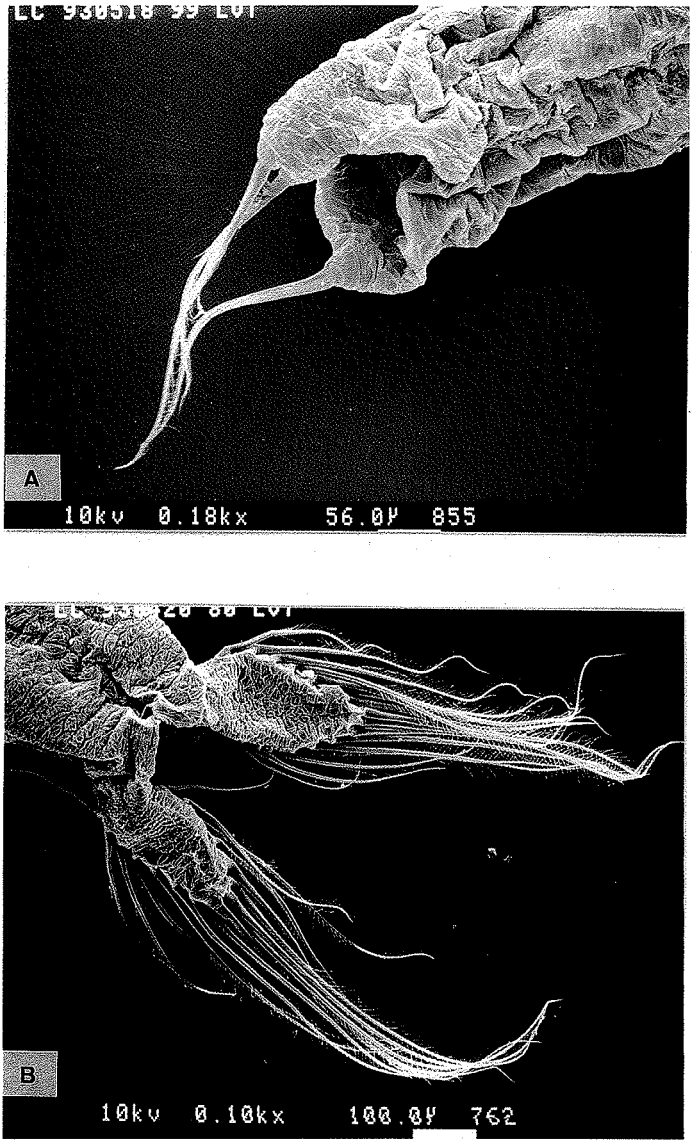

Figure 4. SEM of the differences observed in the length of the furca and number of setae. Plate A: URM population, plate B: KAZ population.

148

Table 6. Mean values (standard deviation in parenthesis) of morphometric and meristic characters of females of different bisexual *Artemia* populations. n = number of animals analysed. Abbreviations of populations in Table 1. A: total length, B: abdominal length, C: length from the third abdominal segment to the end of the abdomen, D: length of the eighth abdominal segment, E: width of third abdominal segment, F: length of furca, G: width of head, H: length of first antenna, I: distance between eyes, J: diameter of complex eye, K: number of setae on the left branch of the furca, L: number of setae on the right branch of the furca, M: width of ovisac. Wilks' lambda (λ) is given by the equation $\lambda = 1 - \eta^2$, where η^2 is the ratio of the between-groups sum of squares to the total sum of squares and represents the proportion of the total variance attributable to differences among the groups (Norusis, 1993)

	SFB $n=30$	GSLI $n=32$	GSLPT $n=31$	YUN $n=30$	YIM $n=30$	INM $n=30$	HAL $n=30$	KAZ $n=31$	SFA $n=31$	MEG $n=30$	URM $n=30$	F ratio	Wilks' lambda	F probability
A	11.14	12.51	10.83	12.53	12.69	11.94	12.48	11.43	10.99	11.32	16.35	104.8313	0.31514	< 0.00005
	(0.64)	(1.01)	(0.71)	(0.97)	(1.18)	(1.05)	(0.96)	(0.96)	(0.87)	(0.71)	(0.87)			
B	5.22	6.27	5.06	6.70	6.89	6.45	6.48	5.89	5.93	6.36	9.89	70.4109	0.23610	< 0.00005
	(0.41)	(0.70)	(0.33)	(0.61)	(0.75)	(0.75)	(0.71)	(0.56)	(0.52)	(0.46)	(0.79)			
C	3.86	4.74	3.66	5.21	5.46	5.03	4.98	4.52	4.67	5.00	8.14	124.2042	0.20689	< 0.00005
	(0.33)	(0.60)	(0.26)	(0.55)	(0.62)	(0.61)	(0.57)	(0.47)	(0.41)	(0.42)	(0.74)			
D	1.13	1.28	1.12	1.51	1.52	1.31	1.38	1.29	1.44	1.56	2.26	64.9692	0.33275	< 0.00005
	(0.12)	(0.18)	(0.12)	(0.21)	(0.19)	(0.20)	(0.20)	(0.17)	(0.17)	(0.20)	(0.27)			
E	0.94	0.87	0.95	0.77	0.76	0.77	0.82	0.73	0.60	0.64	0.70	88.9609	0.26697	< 0.00005
	(0.06)	(0.09)	(0.07)	(0.05)	(0.07)	(0.10)	(0.05)	(0.05)	(0.05)	(0.04)	(0.05)			
F	0.32	0.27	0.33	0.42	0.37	0.45	0.51	0.44	0.36	0.37	0.18	101.5010	0.24197	< 0.00005
	(0.04)	(0.04)	(0.04)	(0.06)	(0.06)	(0.09)	(0.05)	(0.05)	(0.07)	(0.08)	(0.03)			
G	1.17	1.06	1.18	0.99	0.99	1.01	1.07	1.01	0.94	0.92	1.05	35.4556	0.47748	< 0.00005
	(0.07)	(0.08)	(0.07)	(0.06)	(0.08)	(0.17)	(0.06)	(0.06)	(0.08)	(0.05)	(0.05)			
H	0.84	0.88	0.84	1.12	1.13	1.24	1.12	1.33	0.91	0.90	1.19	109.4199	0.22846	< 0.00005
	(0.07)	(0.06)	(0.09)	(0.08)	(0.11)	(0.15)	(0.07)	(0.12)	(0.07)	(0.07)	(0.11)			
I	1.84	1.78	1.86	1.76	1.80	1.78	1.82	1.82	1.62	1.60	2.07	34.0912	0.48728	< 0.00005
	(0.09)	(0.12)	(0.12)	(0.10)	(0.13)	(0.16)	(0.09)	(0.11)	(0.12)	(0.09)	(0.09)			
J	0.33	0.32	0.33	0.28	0.29	0.29	0.31	0.30	0.30	0.29	0.34	18.3637	0.63825	< 0.00005
	(0.02)	(0.03)	(0.02)	(0.02)	(0.02)	(0.03)	(0.02)	(0.02)	(0.02)	(0.03)	(0.02)			
K	10.47	10.56	11.16	10.87	9.97	11.60	13.97	13.93	6.84	7.90	2.10	87.6457	0.26990	< 0.00005
	(1.52)	(2.38)	(2.07)	(2.50)	(2.12)	(3.17)	(1.97)	(2.46)	(1.92)	(2.62)	(1.47)			
L	10.13	10.53	11.22	10.53	9.93	12.23	13.70	13.87	6.84	7.93	2.10	90.2880	0.26408	< 0.00005
	(1.55)	(1.98)	(2.39)	(2.27)	(2.27)	(2.72)	(2.07)	(2.45)	(1.83)	(2.61)	(1.81)			
M	2.19	2.22	2.19	2.13	2.14	2.21	2.06	1.86	1.73	2.04	2.18	14.7967	0.68649	< 0.00005
	(0.22)	(0.27)	(0.20)	(0.19)	(0.23)	(0.34)	(0.21)	(0.18)	(0.23)	(0.21)	(0.26)			

Discussion

In this study we tried to define possible grouping of eleven *Artemia* populations by using the methods of discriminant analysis and hierarchical cluster analysis, based on morphological characteristics. Four distinct groups were identified: the *A. franciscana* group which is composed of the SFB, GSLI and GSLPT populations, the *A. tunisiana* group represented by the SFA and MEG populations, the *A. urmiana* group with only one population (URM) and a larger group which includes the Eastern Old World (EOW) populations YUN, YIM, INM, HAL and KAZ.

The statistical analyses on the biometry of both males and females revealed that in the *A. franciscana* group the GSLPT strain is morphologically closer to the SFB than to GSLI population. Due to the lack of information about the exact origin of these strains (commercial sources) it is not possible to say that the SFB strain seems to present greater similarities with GSLPT than that observed between the two GSL strains. Alhtough the above observations seem to contradict the data presented by Hontoria & Amat (1992b), we think that this is not true, since the number of the *A. franciscana* populations studied in our case is smaller. Here, we may refer to the similarities one can observe between the two habitats (SFB and GSL) where chloride is the prevailing anion (Bowen et al., 1985; 1988).

Both representatives of the Mediterranean bisexual species *A. tunisiana* (MEG and SFA populations)

Table 7. Significant differences for the mean values of morphometric and meristic characters of bisexual females. The differences determined by Anova and the Tukey's B test (Norusis, 1993) when the Levene's test showed homoscedasticity of the variances; when variances were heteroscadastic the approximate test of equality of means of Games & Howell (1976) was employed. Populations that share the same letter (a, b, . . . , f) per row, are not significantly different (a = 0.05). The asterisk denotes the characters that the Levene's test showed departure from homoscadasticity. The codes (A, B, . . . , M) for the various characters are the same as in Table 6. The abbreviations of populations can be found in Table 1

	SFB	GSLI	GSLPT	YUN	YIM	INM	HAL	KAZ	SFA	MEG	URM
A	a	c, d	a	c, d	d	b, c	c, d	a, b	a	a, b	e
B	a	b, c	a	c, d	d	c, d	c, d	b	b	c	e
C	a	b, c	a	d, e	e	c, d	c, d	b	b, c	c, d	f
D	a	b	a	c, d	c, d	b	b, c	b	c, d	d	e
E*	d, e	d	d, e	b, c	b, c	b, c	c, d	b, c	a	a	b
F*	c	b	c	d	c	d, e	e	d	c	c	a
G	e	c, d	e	b	b	b, c	d	b, c, d	a	a	c, d
H*	a, b	a, b, c	a	d, e	d, e	e, f	d, e	f	a, c	a, b, c	d, e, f
I*	b, c	b, c	b, c	b	b, c	b, c	b, c	b, c	a	a	d
J	e, f	d, e	e, f	a	a, b, c	a, b	c, d	b, c, d	b, c	a, b, c	f
K*	c	c	d	c	c, d	d	e	d	b	b, c	a
L*	c, d	c, d	d, e	c, d	c, d	d, e	e	e	b	b, c	a
M	b	b	b	b	b	b	b	a	a	b	b

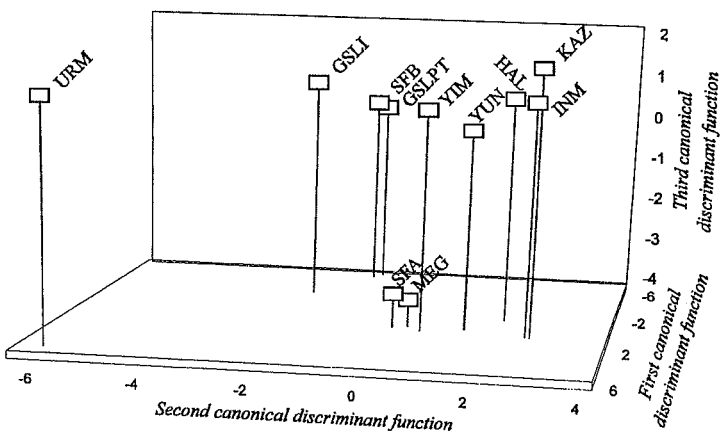

Figure 5. Scatterplot of the first three canonical discriminant functions (group centroids) resulting from the discriminant analysis on females when using the origin of each population as separation factor. For legend to abbreviations see Table 1.

showed morphological similarities with the EOW populations. Multivariate analysis of male and female morphological characters revealed that the URM population appeared to be more 'isolated' from the other populations.

In the EOW group the strains YIM, YUN and INM seemed to be closer and formed a subgroup, while the HAL and KAZ populations appeared to be more distinct (Figures 1, 2, 5 and 6). YUN population, which belongs to *A. sinica* (Cai, 1989; Pilla, 1992), presents significant similarities with the YIM and INM popula-

150

Table 8. Results of the discriminant analysis for females. The classification was based on the origin of each population

Variables	Unstandardized canonical discriminant function coefficients*			Standardized canonical discriminant function coefficients*		
	1	2	3	1	2	3
Total length	-6.2540889	-16.7697280	-2.5548048	-0.20494	-0.54952	-0.08372
Abdominal length	18.1499524	-11.3555652	-11.0416864	0.74015	-0.46308	-0.45028
Length 3rd abdom. seg.	2.8097282	13.3402151	22.9098474	0.12391	0.58833	0.01036
Length 8th adom. seg.	-1.7405557	-1.0942844	-8.3798847	-0.09872	-0.06206	-0.47527
Width of 3rd abdom. segm.	-21.8220439	1.2558924	20.9047153	-0.79963	0.04602	0.76601
Length of furca	0.8036411	10.3155543	-7.6058528	0.05473	0.70255	-0.51800
Width of head	11.6484444	-0.7937181	-2.2059805	-0.37301	-0.02542	-0.07064
Length of 1st antenna	17.3210356	11.2102084	13.4042069	0.66836	0.43256	0.51722
Distance between eyes	7.8036127	-8.9346394	15.1537565	0.21665	-0.24806	0.42072
Diameter of eyes	-1.3403172	-5.9233560	-7.4003101	-0.04773	-0.21094	-0.26354
No. of setae left branch	-0.3578187	0.0800649	0.7078144	-0.13914	0.03113	0.27523
No. of setae right branch	-0.00886343	0.5172304	0.6736415	-0.00342	0.19947	0.25979
Width of ovisac	-0.1703540	2.7137695	-9.7653978	-0.00843	0.13436	-0.48349
Constant	-13.1272580	19.4761306	-12.9715381			

	Eigenvalue**	Percentage of variance	Cumulative percentage	Canonical correlation	Wilks' lambda***	Chi-square	DF	*P*
Function 1	11.3064	50.30	50.30	0.9585	0.000537	2424.565	130	< 0.0001
Function 2	5.9620	26.53	76.83	0.9254	0.006607	1616.306	108	< 0.0001
Function 3	2.9540	13.14	89.97	0.8643	0.046000	991.475	88	< 0.0001

* The unstandardized coefficients are the multipliers of the variables when they are expressed in the original units, while the standardized coefficients are used when the variables are standardized to a mean of 0 and a standard deviation of 1, just like in multiple regression (Norusis, 1993).

** Eigenvalue is the ratio of the between groups to within groups sums of squares. Large eigenvalues are associated with 'good' functions (Norusis, 1993).

*** See Table 6.

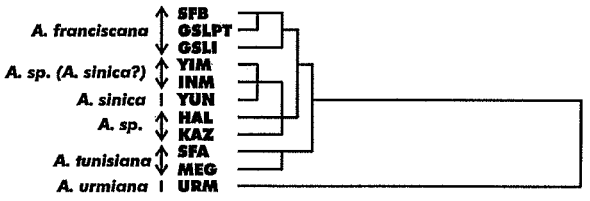

Figure 6. Dendrogram of hierarchical cluster analysis for females using the single linkage method to join clusters (nearest neighbor technique). For legend to abbreviations see Table 1.

tions. Taking into account: (1) that they are geographically close and (2) that they allow successful interbreeding under laboratory conditions (Pilla, 1992), there is good evidence that these populations (YIM, YUN and INM) might belong to the same species. Pilla (1992) and Pilla & Beardmore (1994) comparing the KAZ population with the species *A. urmiana* and *A. sinica* (from Yuncheng) concluded that 'there is no apparent large-scale congruence between genetic, geographic and morphometric distances between these three populations'. In agreement with the previous studies, the KAZ population appears to be clustered separately from the populations of YUN, INM and YIM and this is more evident in males (Figures 1 & 5).

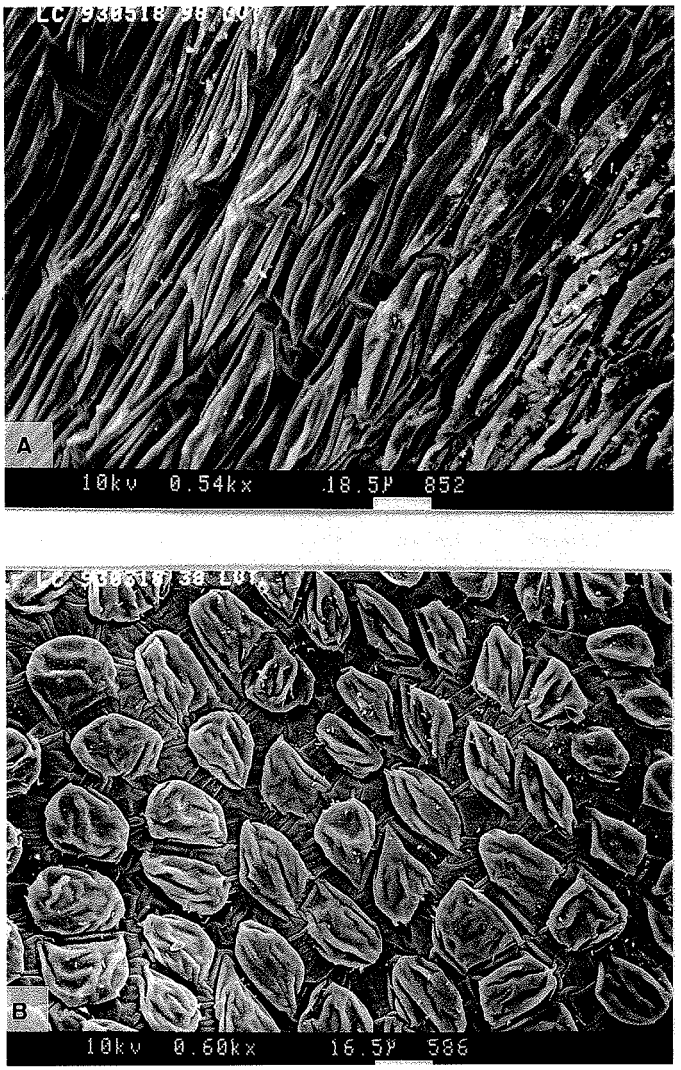

Figure 7. SEM of the differences observed in the pattern of the ectodermal ridges of the brood pouch. Plate A: URM population. Plate B: INM population.

The HAL population is always grouped between KAZ and the *A. sinica* group. Genetic studies showed that the HAL population presents significant deviations from Hardy-Weinberg equilibrium, suggesting that it might be a mixture of two populations (Kathleen Thomas, University of Wales, personal communication) which might be a possible explanation according also to our data.

152

Our results showed that although a high prediction can be obtained for a particular population, it is rather difficult to obtain 100% prediction of group membership in populations belonging to the same species, which is in agreement with the results of Hontoria & Amat (1992a). It is evident that the great genetic similarities of populations belonging to the same species are reflected in their morphology.

Although morphological differences between males are not obvious by simple allometric observation, they become clear after applying statistical multivariate tests. In agreement with Pilla (1992) and Pilla & Beardmore (1994) this study shows that male morphological characters are very useful and should be considered in a morphological characterization of a bisexual population. In our case, overall discrimination based on male characters was not better than that based on females, as Pilla & Beardmore (1994) found in their study on *A. urmiana*, *A. sinica* and a population from Kazakhstan. These differences in the morphology of the adult males might eventually make it possible to discriminate rare parthenogenetic males (Bowen et al., 1978; MacDonald & Browne, 1987).

Scanning electron microscopy study showed that apart from obvious differences in the morphology of the telson and the number of setae, assigning obvious morphological differences between the various populations is rather difficult. Earlier studies (Mura et al., 1989a,b) showed that two main patterns of frontal knob morphology can be distinguished: subspherical knob pattern that characterizes the species *A. franciscana* and *A. persimilis* and subconical knob pattern for *A. tunisiana* populations. In our study it proved difficult to assign a particular subconical or subspherical knob pattern and in agreement with Thiéry & Robert (1992) we believe that frontal knobs are of insufficient taxonomic value without further statistical study.

The presence of a pair of spine-like outgrowths at the basal part of the penes of all the studied populations and their absence from the SFA population seems to be a good taxonomic character for the discrimination of *A. tunisiana* populations from other species in the genus.

The pattern of the ectodermal ridges of the brood pouch of URM showed marked differences compared to the other populations and it should be considered as a character with discriminating power whenever *Artemia* samples from Urmia lake or other geographically-close lakes are characterized.

In most morphological studies, different culture conditions have been used, making the comparison of the results difficult. We would like to propose the use of standard culture conditions for similar studies in the future.

Conclusion

The use of multivariate procedures gives strength to the classic method of morphological strain characterization. It can be used for preliminary strain classification and for detailed characterization of bisexual *Artemia* populations. The method of discriminant analysis and hierarchical cluster analysis clearly separates and groups together populations which exhibit great genetic similarities.

Acknowledgements

This research is financed by project TS2-CT91-0331 of the European Union and is a collaboration of the Universities of Ghent (Belgium), Swansea (United Kingdom), Milan (Italy) and the Salt Research Institute (P. R. China). G.V.T. is a scholar of the 'Alexander S. Onassis' and 'Empirikion' Public Benefit Foundations (Greece). We thank James de Caluwé for introducing us to the Windows working environment and for his help to prepare the graphs. We also express our thanks to an anonymous reviewer for his helpful suggestions that improved our paper.

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